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Short report

Safeguarding forensic DNA reference samples with nullomer barcodes

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ABSTRACT

Unintended transfer of biological material containing DNA is a concern to all laboratories conducting PCR analysis. While forensic laboratories have protocols in place to reduce the possibility of contaminating casework samples, there is no way to detect when a reference sample is mislabeled as evidence, or contaminates a forensic sample. Thus there is public concern regarding the safeguarding of DNA submitted to crime labs. We demonstrate a method of introducing an internal amplification control to reference samples, in the form of a nullomer barcode which is based upon sequences absent or rare from publically accessible DNA databases. The detection of this barcode would indicate that the source of analyzed DNA was from a reference sample provided by an individual, and not from an evidence sample. We demonstrate that the nullomers can be added directly to collection devices (FTA paper) to allow tagging during the process of sample collection. We show that such nullomer oligonucleotides can be added to existing forensic typing and quantification kits, without affecting genotyping or quantification results. Finally, we show that even when diluted a million-fold and spilled on a knife, the nullomer tags can be clearly detected. These tags support the National Research Council of the National Academy recommendation that "Quality control procedures should be designed to identify mistakes, fraud, and bias" in forensic science (National Academy of Sciences, 2009).

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1. Introduction

Unintended transfer of biological samples is an issue of great concern to all laboratories conducting sensitive analyzes. This is particularly true for crime laboratories, where victims, suspects, and even investigators are asked to supply reference samples of their DNA for comparison to evidence profiles. Contamination, or unintended transfer of DNA, can happen at any time in the process of forensic DNA analysis. Instances of unintended transfer and mislabeling by personnel collecting and processing samples, while uncommon, have been documented,[1–3] and concern about supplying reference samples has been raised by individuals and groups including those representing police officers in Connecticut and Missouri.[4,5] This problem is likely to become more pronounced as forensic DNA techniques become ever more sensitive, and as databases grow with the increasing use of forensic DNA.[6] While forensic DNA analysis is considered the "gold standard" of forensic science practice, there is the expectation that the best possible practices of regulation and oversight be implemented. With regard to the forensic sciences in general, the 2009 report by the National Research Council of the National Academy recommends that "Quality control procedures should be designed to identify mistakes, fraud, and bias" as well as "confirm the continued validity and reliability of standard operating procedures and protocols."[1] With this in mind, while recognizing that laboratory mistakes and mishandling are rare events, we present a means by which to provide an additional "safeguard" for reference samples used in forensic DNA analysis, employing sequences known to be rare or absent in nature[7,8] as internal controls. These sequences (known as nullomers) could be used to both verify sample origin (as a reference sample, or any sample not collected from a crime scene) and as way to potentially track contamination if it occurs.

Studies have shown the propensity of DNA to be deposited by individuals via the mere touching of objects, such as DNA from fingerprints[9] or the secondary transfer of touch-deposited DNA.[9–11] Ladd et al. (1999) found an average of 1–15 ng DNA recovered from touched objects (dependent on the object).[10] Assuming that 1 ng of genomic DNA is the equivalent of genomes from ~170 cells,[12] the

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swabs in the experiments of Ladd et al. picked up the DNA from ~170 to ~2500 cells. With modern techniques that can produce profiles from a few or even single cells,[13] it is expected that the detection of DNA from secondary and tertiary transfer will become more common. In the context of clinical genetic testing, a case of DNA contamination has been reported, and routine forensic STR typing was recommended to ensure that all DNA samples are truly from a single individual.[14] Contamination of samples with amplified products has also been a concern, recognized since the early days of PCR.[15] Laboratories which use enhanced techniques for low template DNA analysis have detected contamination in reagents, plastic ware and laboratory samples at levels that are below detection with less sensitive methods.[16] While most crime labs have protocols in place to prevent PCR products from coming in contact with pre-amplified DNA samples, accidents can happen. Unfortunately, it is not presently possible to detect when a reference sample contaminates an evidentiary sample. A specific incident mentioned in the *Final Report of the Independent Investigator for the Houston PD Crime Lab* documents case in which a re-analysis indicated "that the victim's reference sample was contaminated at some point in the handling of this sample."[3] The independent investigator concluded that the contamination probably occurred after extraction, and happened "most likely at the PCR amplification stage".[3]

In order to safeguard against the accidental transfer or contamination of DNA samples collected from members of the public, we have developed a unique type of internal amplification control (IAC) that can serve as a universal tag and barcode and can be modified to further encode a variety of information, such as laboratory location, testing purpose, or date. These tags are based on the smallest sequences absent from all publicly available DNA databases (nullomers), first described by Hampikian and Andersen,[7] and nullomer technology is also being used to design small peptide drugs.[8] These nullomer IACs (also designated "nullomer barcodes") can be embedded in collection devices such as swabs and FTA paper, thus allowing DNA samples to be tagged at the earliest stage in the DNA analysis process. The nullomer approach has generated recent interest in algorithms for counting and tracking biological sequences.[17,18] This paper describes, to the best of our knowledge, the first practical forensic application of nullomer sequences. Our results show that nullomer sequences can be used as an IAC, and as molecular tags and barcodes, successfully integrated into the multiplex PCR reactions of commercially available forensic profiling kits, and used along with PCR for sequencing. The use of IACs (sometimes designated internal positive controls, IPCs) is already commonplace for food and clinical microbiological testing,[19] forensic quantification kits,[20] forensic human identification kits,[21–23] and are particularly useful for detecting PCR inhibitors.[24] For PCR-based tests of food-borne pathogens, the European Standardization Committee has developed guidelines that require the presence of an IAC.[25] The nullomer approach described here is unique in two important ways: it can be used to distinguish between reference and evidentiary samples, and the tags are designed through an algorithm which identifies small sequences absent from the public databases of all sequenced organisms.[8]

2. Methods

2.1. Nullomer sequences and primer design

The algorithm of Hampikian and Andersen[7] is able to process the entire set of biological sequence data found on NCBI's website in less than 8 h, calculating the frequencies of all sequences up to length 17 (longer lengths can be calculated by our methods as well). Basically, all possible sequences up to a given length are generated, and each sequence is compared to those in the databases; any sequences that are not found in the databases are listed as nullomers. At length 17 there are (as of January 2011) approximately 700 million (695,038,288) absent sequences in the NCBI data bases.

From a list of absent 15-mers, we concatenated eight sequences to form a 120 bp nullomer "tag". Several permutations of eight 15-mers were analyzed via the Oligoanalyzer tool available at the Integrated DNA Technologies (IDT) website (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Sequences were chosen to minimize the formation of secondary structure, and primer-binding regions were chosen so that the annealing temperature would match as closely as possible the 59 °C annealing temperature of the ABI kit protocols (58 °C for Powerplex™ Y kit). Most nullomer sequences have a high GC content; therefore the list of potential 15-mers was reduced to those having a GC content in the 40–60% range. However, since the primer binding region of the sequence is the determining factor in PCR specificity, the internal sequence is amenable to sequence modification, allowing nullomer tags to be used for DNA "cryptography" or barcodes. The sequences of the barcode primers used are shown in the supplementary material. The 120 bp construct was synthesized by IDT (Coralville, IA, USA) as two complimentary single stranded molecules. This complimentary pair was annealed to make a double stranded oligomer. After annealing, remaining single stranded molecules were removed with ExoSAP™ enzyme treatment. Concentration of nullomers (copies/μL) was calculated by measuring ds DNA concentration on a NanoDrop spectrophotometer, and calculating expected copy number from the molecular weight of each nullomer molecule. Primers were designed to yield amplicon sizes of 88, 90, and 114 bp. Several barcode concentrations were tested with different STR kits; we present the main results using 1900 copies per PCR reactions (3800 copies used in the Identifiler™ experiment shown in Fig. 1b), which gave barcode peaks at intensities comparable to the human STR alleles. In order to visualize the amplified nullomer peak on the 3130 Genetic Analyzer, the nullomer primers were ordered from IDT with a 6-FAM modification to the 5′ end of one of the primer pair. In our experiments, we found that a rather high concentration of primers (2.5 μM) was useful for amplification of the nullomer tag such that the nullomer peaks approximated the size of normal amplicon peaks. Although higher than the standard range for PCR primers (0.1–0.5 μM), we saw no evidence for primer-dimer formation or non-specific amplification.

2.2. Co-amplification of forensic loci and nullomer tags

We designed the tag (barcode) DNA amplicons to be of a size outside the range of human STR alleles, so that there is no confusion between the barcode and known STR allele peaks. Human DNA and barcode DNA was amplified according to manufacturer protocols (except for the addition of the nullomer barcode and barcode primers) with the following forensic DNA kits: Quantifiler Duo™ (Fig. 1a), Identifiler™ (Fig. 1b, Fig. S1–S4), Profiler Plus™ (Fig. S5 and S6), Yfiler™ (Fig. S7), and PowerPlex ™ Y (Fig. S8 and S9).[26–30] To test nullomer tag compatibility with mitochondrial DNA (mtDNA) sequencing, we amplified the HV-1 and HV-2 regions of human mtDNA in the presence of nullomers, with and without tag primers. Results from the Quantifiler Duo™ test, as well as results with Identifiler™ (performed in triplicate), were analyzed with unpaired *t*-tests and a one-way ANOVA, using GraphPad Prism version 5.00 for Windows, to test for significant differences, $p \leq 0.05$.

A common technique for the storage and processing of reference samples is to transfer a buccal swab sample to FTA paper and allow it to dry. DNA samples fixed onto FTA paper can be stored at room temperature, and then be extracted from FTA paper punches when needed. We treated an FTA card with a solution of nullomer DNA in nanopure H_2O, allowed it to dry, and then transferred DNA from a buccal swab to the FTA card.

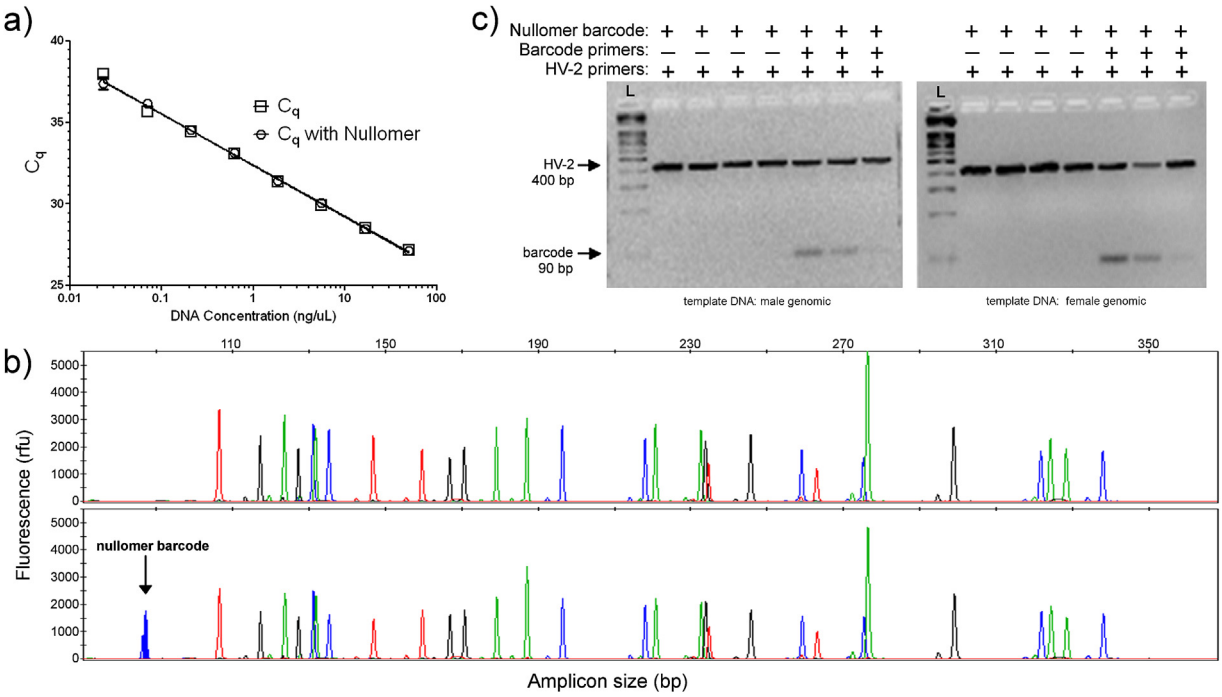

Fig. 1. a. Nullomer tag does not interfere with Quantifiler DUO™ DNA quantification. A standard curve was generated using control DNA supplied with the Quantifiler DUO™ kit from applied biosystems. The DNA standard was diluted, and the real-time PCR performed, according to the manufacturer protocol. Three sets of standards were made without the nullomer tag, and three sets were made with nullomer tag added to the reaction mix. Number of cycles to reach the quantification threshold (C_q) is shown, for DNA with (○) and without (□) 1.9×10^3 copies of nullomer barcode. Results show mean ± SEM. b. STR profile of female genomic DNA in the presence and absence of the nullomer barcode, amplified with the Identifiler™ kit. Top electropherogram, STR amplification without the nullomer barcode. Bottom electropherogram, STR amplification in the presence of nullomer barcode (3.8×10^3 copies). Size of each amplified product is given in base pairs; the locus is indicated by labels above the peaks. The *y*-axis is in RFUs, and is scaled according to maximum peak height. The nullomer peak is the smallest fragment (90 bp amplicon). c. HV2 region of mitochondrial DNA from male (left) and female (right) amplified in the presence and absence of the nullomer barcode. Mitochondrial PCR product was visualized on a 3% agarose, ethidium bromide stained gel. HV2 product amplified properly with nullomer barcode (with and without nullomer primers added to the HV2 PCR reaction).

2.3. Mock contamination experiments with nullomer-tagged reference and post-amplification samples

We setup a mock unintended transfer of Identifiler™-amplified DNA (with nullomer barcode and nullomer primers co-amplified) to a mock evidentiary weapon (knife). The amplified DNA (~0.5 μL) was allowed to dry, and the knife swabbed later with a wet sterile Omni-swab (Whatman). DNA was extracted from the swab and analyzed with the Identifiler™ kit, with nullomer primers added to the PCR reaction.

Another mock contamination experiment was performed, with barcode-tagged reference DNA mixed with a forensic DNA extraction from the surface of a plastic coffee cup lid. A nullomer tag was added to a sample of "reference DNA" extracted from the buccal swab of a volunteer (reference DNA concentration was 36 ng/μL, as measured with a Nanodrop Spectrophotometer). The reference DNA sample was augmented with ~80,000 copies of barcode (~2000 copies of nullomer barcode per μL of reference DNA), and then 1.0 μL of tagged reference sample was added to the forensic swab, prior to DNA extraction. The tagged DNA was extracted along with the forensic sample, to simulate a contamination event involving reference DNA. The nullomer primers used for this experiment generated a 113-bp amplicon.

3. Results

Adding the nullomer tag to a human buccal swab did not affect the quantification of extracted DNA (Fig. 1a). The kit used in our experiment (Quantifiler® Duo) is commonly employed by forensic laboratories to determine the DNA concentration for both the total human and male fraction (Y-chromosome) of a sample. We demonstrate that the nullomer tag does not negatively impact quantification of either total human or male fraction DNA (unpaired *t*-test, $p \leq 0.05$).

When amplified with the STR alleles of the forensic kits we tested, the 90 base pair nullomer barcode appears in the electropherogram as an additional peak outside the first "bin set" (regions where peaks from alleles from the various loci are known to occur). There were no differences in the DNA profile of individuals when barcode DNA was added. The nullomer DNA does not interfere with STR genotyping of individuals (Fig. 1b).

When we extracted and amplified DNA from nullomer-treated FTA paper, we obtained a profile which includes the barcode tag, identifying the DNA as coming from a reference source (Fig. S10), and not from evidence.

Barcode PCR products were co-amplified with mtDNA targets (when barcode primers were added to the reactions), and detected as distinct bands of ~90 bp (Fig. 1c, Fig. S11). Sanger sequencing of the amplified mtDNA was not affected by the presence of the nullomer barcode, whether the barcode was added during initial PCR using HV1 and HV2 primer sets, or if added to the sequencing reactions using HV1 or HV2 amplicons as templates (Fig. S13).

Amplification of barcode DNA along with human DNA in an Identifiler™ reaction did not adversely affect the amplification of the forensic loci, as shown by a comparison of mean peak heights (±SEM) for each allele, with and without the nullomer tag added and amplified (Fig. 2).

The analysis of the DNA collected from the mock forensic contamination event showed a clear signal of nullomer DNA, at two different dilutions. A 10^5-fold dilution of the PCR product showed

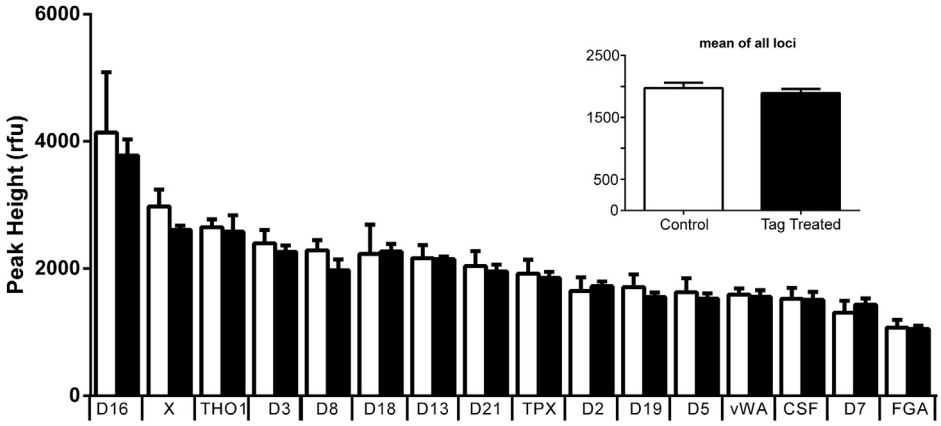

Fig. 2. Average peak heights (per locus) of human DNA amplified with the Identifiler™ kit, with and without nullomer barcode and FAM-labeled primers added. Experiment performed in triplicate. Results shown ± SEM. White bars: control. Black bars: with 3800 copies of nullomer barcode-1 added. Inset: average peak heights of all alleles. No significant differences between control and treatment peak heights, per locus, and over all loci (unpaired *t*-test, $p \leq 0.05$).

amplification of nullomer barcode DNA along with a partial profile of the transferred human amplicons (Fig. 3, top electropherogram). After a 10^6-fold dilution, the nullomer barcode could be detected; even though the human profile was lost at our signal threshold of 100 RFUs (Fig. 3, lower electropherogram, Fig. S13).

Nullomer-tagged reference DNA can be detected when it contaminates a forensic DNA sample (Fig. 4a). Allelic drop-out was observed in this experiment, as is commonly seen in amplifications of low levels of DNA, but there was no correlation with barcode treatment. The amplification of the contaminated DNA mixture was carried out 5 times with and without nullomer barcode primers. A typical example with the barcode primers is shown (Fig. 4a), and two amplifications of the same extract without barcode primers (Fig. 4b, c). Some allelic drop out occurred whether or not the nullomer barcode was amplified (Table 1), however more extensive validation will have to be performed to optimize and validate particular nullomer tags.

We have shown that an artificial DNA barcode can be used in conjunction with forensic genetic analysis kits, without affecting DNA quantification, STR amplification, profile determination, or

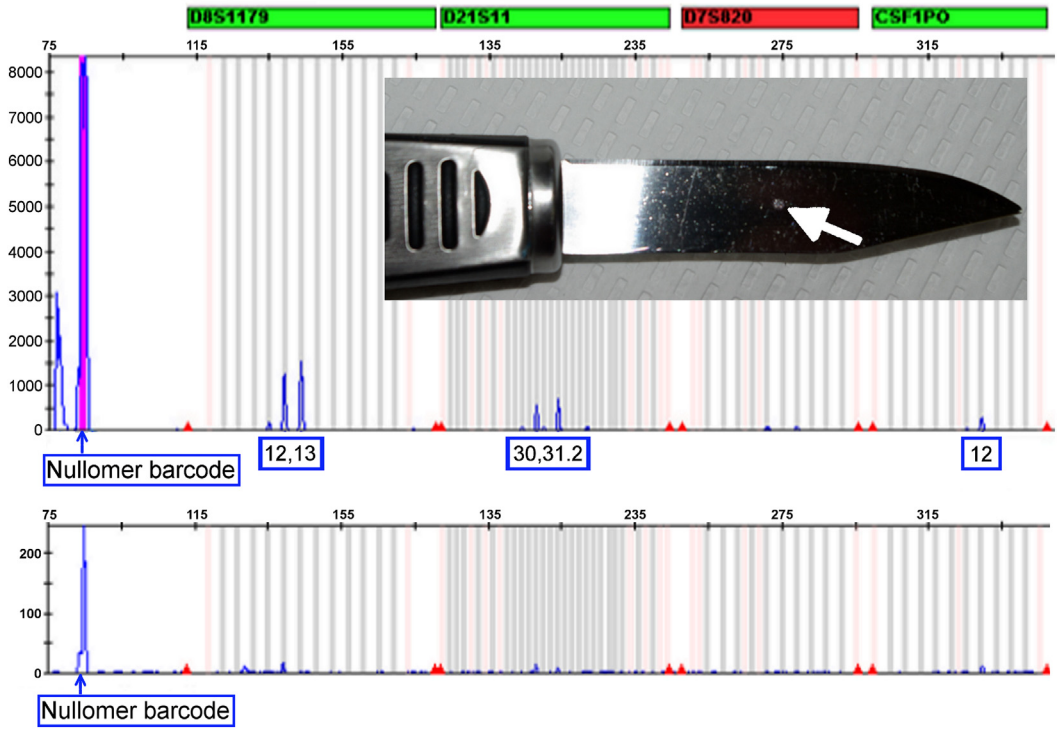

Fig. 3. Contamination of evidence with amplified DNA is detected with nullomer barcode, even when diluted 1,000,000 fold. Top panel and inset: DNA from amplified STR profile (amplified with nullomer barcode) was diluted 100,000 fold in water, and then 1ul of the dilution was applied to a newly purchased knife. This knife was swabbed, and amplified according to the STR kit manufacturer's instructions (with the addition of nullomer primers). The STR profile from the contaminated knife shows both the contaminating profile, and the nullomer barcode peak. Bottom panel electropherogram: original PCR product was diluted 1:1,000,000 in water, and 1 μL of the diluted product was added to a newly purchased knife. The knife was swabbed and processed as above. The nullomer barcode amplicon is prominent, although no alleles from the amplified human profile are detected at this dilution.

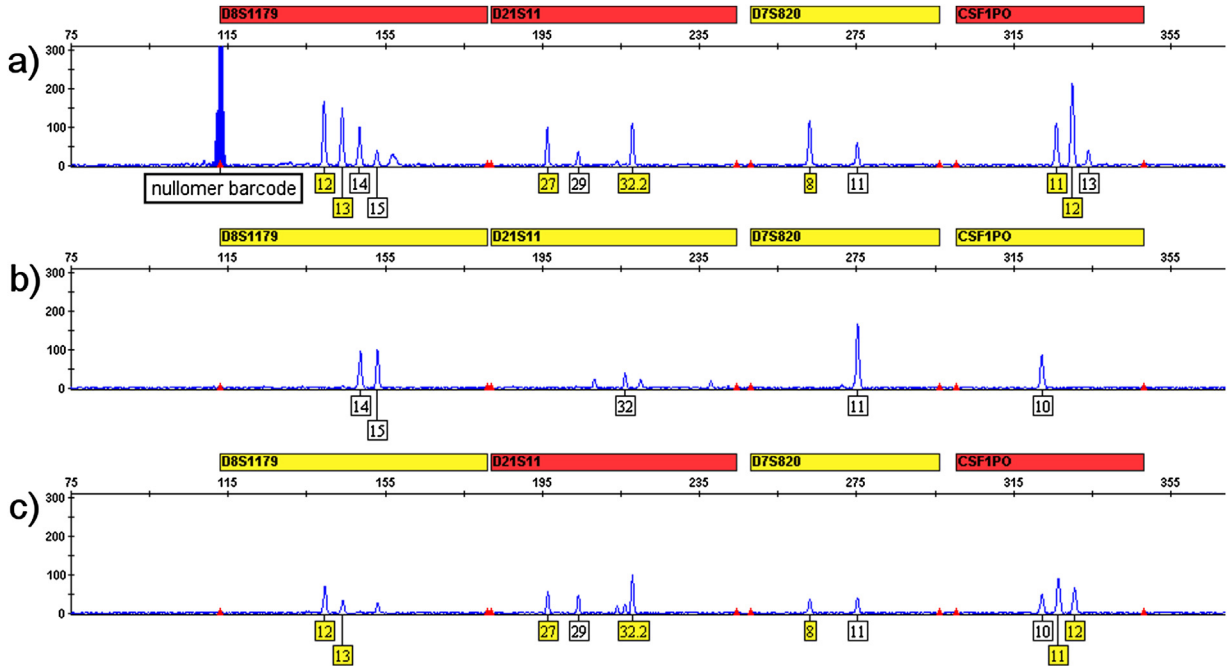

Fig. 4. Contamination of forensic DNA sample with reference DNA is detected with nullomer barcode amplification. a. Identifiler™ profile (blue channel) of DNA from coffee cup (forensic sample) that was contaminated with reference DNA. The presence of nullomer tag peak indicates that a contamination event took place. Allele numbers highlighted in yellow indicate the contaminating reference DNA. b and c. Identifiler™ profile of same mixture, except that nullomer primers were not added to the PCR reaction. Note that stochastic effects from low amount of template DNA result in allele drop-out, with or without nullomer amplification (see text for more details).

mitochondrial sequence, using standard protocols. DNA profiles were obtained by amplification of 0.5–1.0 ng of genomic DNA in the presence of 1900–3800 copies of nullomer barcodes (as indicated). We have stored the barcodes at 4 °C for one year in TE buffer without affecting amplification and detection, and used FTA paper with dried barcodes for six months without any noticeable effect on amplification.

4. Discussion

Extrinsic DNA can enter the laboratory through contaminated reagents, disposables, centrifuges, and water baths.[15,16,31] Reagent controls and routine monitoring can detect many of these examples, however, contamination of evidence with DNA from reference samples taken from suspects, or the switching of reference samples, is more difficult to detect, and may in fact implicate an innocent person in a crime.[2] The nullomer tags described in this paper were developed to assure the public that their reference samples can be marked so as to prevent false incrimination. Further development of the tag technology can be used to code individual samples to further safeguard the public.

The initial tags described here could be added to collection kits so that reference samples are "safeguarded" from the point of collection. Physical and chemical modifications of the tags could be used to stabilize them further[32]; though we demonstrate here that even unmodified synthetic DNA is sufficient.

Synthetic DNA barcoding has been used to make positive amplification controls for applications in a wide variety of fields, such as clinical microbiological testing[33] and food pathogen testing,[19] and has been proposed for a variety of tagging and tracing protocols.[34,35] Internal positive controls are already an important part of forensic genotyping and qPCR quantification kits.[20,23–30] In those kits, the IPC allows the forensic analyst to assess the presence of contaminating PCR inhibitors, such as hematin or humic acids.[25] Unlike our nullomer tagging protocol, current tags are present in the PCR reaction components, and so cannot distinguish reference from evidentiary samples. IPCs currently used in forensic kits have been designed such that the IPC sequence was checked against GenBank to ensure uniqueness—but the methods of sequence design are not always reported.[36] Non-human sequences have been used for this purpose, such as a portion of Sea Pansy (*Renilla reniformis*) luciferase gene,[37] a hydra-specific (*Hydra vulgaris*) actin gene segment,[22,23] or rat DNA.[38] In some commercial kits (e.g. the Quantifiler® Duo kit), the IPC is a trade secret and is simply designated a "synthetic polynucleotide" in the published literature.[20] Our approach of building from these small absent sequences is novel. While these sequences may eventually appear in a natural database, it is highly unlikely that concatamers based on them will—and for such a

Table 1
No significant difference in detectable alleles with or without Nullomer barcode in a low template DNA sample. Stochastic allelic drop out in 5 Identifiler runs with and without nullomer barcode primers. Results are shown for the cup mixture profiles (Fig. 4) using Identifiler, with and without barcode primers. The detection threshold of 35rfu cut-off was used for this low template DNA analysis. No significant difference between treatments was found, using χ^2 test; $p \leq 0.05$, df = 13. D7 and Amel were not used in χ^2 analysis (zero values).

# Alleles dropped out	D8	D21	D7	CSF	D3	THO1	D13	D16	D2	D19	vWA	TPOX	D18	Amel	D5	FGA
Without nullomer primers	4	6	0	5	4	2	3	5	8	4	5	3	6	0	5	4
With nullomer primes	0	4	0	5	2	4	3	3	7	4	1	1	6	0	4	5

sequence to arise and work with our primers, and produce the proper sized amplicon, would be quite remarkable. An easy verification for checking that the peak does correspond to the correct nullomer tag would be sequencing the PCR products with barcode primers, which would reveal the exact nullomer sequence in a tagged sample. While we don't envision sequencing every reference sample, the nullomer tags could be used to trace the source of contamination. This should be a rare event, but it would be valuable to trace the origin of suspected contamination. The nullomer barcode that we have designed is 120 bases long, and since only ~1900 copies are needed per PCR reaction, the additional cost to sampling kits would be minimal. These barcodes could easily be added to FTA paper, liquid buffer, cotton swabs, or other components of human DNA sampling kits. While DNA has the power to free the wrongfully convicted,[39] it can also be the route of forensic error as illustrated by a recent, highly publicized wrongful conviction and incarceration, due to mislabeling of DNA samples.[2] As DNA sampling and archiving becomes routine, the public needs to be assured that their DNA is being properly collected, stored and interpreted.[1,5,40,41] We propose that nullomer markers can support the National Research Council's recommendations for strengthening and improving forensic science in the United States.[1]

Ethical approval

None.

Financial support

None.

Conflict of interest

One of the authors (Greg Hampikian) has also applied for a patent covering the nullomer applications described in this article.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jflm.2013.02.003.

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